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The human *LINE-1* retrotransposon (*L1Hs*) is known to cause mutations by inserting into genes and inactivating them. The element is expressed in many breast tumors and breast tumor cell lines, suggesting that *L1Hs*-induced mutations may play some role in this malignancy. To test this hypothesis we plan to place a "tagged" *L1Hs* element in non-malignant breast epithelial cells and let it transpose. If the cells become malignant, we can isolate the gene(s) into which the element has transposed by using the unique tag. To date we have tested a number of vectors in several cell lines, but attempts to obtain stably transfected cells expressing *L1Hs*-encoded proteins have not been successful. We are currently exploring the use of other cell lines that may increase our chance of success.

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FOREWORD

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Thomas C Tenney Oct, 24, 1996

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Abstract

The human *LINE-1* retrotransposon (*L1Hs*) is known to cause mutations by inserting into genes and inactivating them. The element is expressed in many breast tumors and breast tumor cell lines, suggesting that *L1Hs*-induced mutations may play some role in this malignancy. To test this hypothesis we plan to place a "tagged" *L1Hs* element in non-malignant breast epithelial cells and let it transpose. If the cells become malignant, we can isolate the gene(s) into which the element has transposed by using the unique tag. We have tested a number of plasmid and retroviral vectors in several cell lines without success. However, one expression vector, pIRES1neo (producing a bicistronic mRNA), has given stably transfected MCF10A cells expressing our "test" protein (the *L1Hs*-encoded p40 protein). The amounts of this protein can be controled by external agents that induce the cytomegalovirus promoter/enhancer which drives transcription of the construct. We are currently placing the "tagged" *L1Hs* element into this vector so that we may proceed with Aim 1 of the grant.

Introduction

Human *LINE-1* (*L1Hs*) is a transposable element that encodes a reverse transcriptase and moves *via* an RNA intermediate [1]. It therefore seems possible that cells in which *L1Hs* is active may be subject to insertional mutagenesis. We have recently found that the element is expressed in a significant number of germ cell cancers [2,3], and in many breast tumors and breast tumor cell lines [4,5]. These findings raise the possibility that the initiation or progression of malignancy in certain steroid hormone responsive tissues is facilitated by *L1Hs* expression and/or transposition.

In addition to insertional mutagenesis, there are several other characteristics of the *L1Hs* element that suggest its potential as an oncogenic agent. For example, *L1Hs* has an internal promoter which could potentially lead to readthrough transcription and activation of downstream genes. In addition, the *L1Hs*-encoded p40 protein has a leucine zipper motif, suggesting a possible interaction with other cellular proteins. Such interactions at inappropriate times might lead to the disruption of important cellular functions. Thus, *L1Hs* involvement in cancer could occur by several mechanisms, either singly or in combination.

Our long range goals focus on the isolation of cellular genes that are affected by *L1Hs* transposition. These genes are presumably the ones whose inactivation (by insertional mutagenesis), or activation (by readthrough transcription), is one of the steps in the pathway leading to malignancy. Specifically we have proposed to:

(1) Place a "tagged", transpositionally competent *L1Hs* element into non-malignant human breast epithelial cells.

(2) Identify malignant cells arising from the non-malignant cell population and isolate and characterize sequences into which the tagged *L1Hs* element has transposed.

Experimental

In our initial experiments we have used vectors containing only the first *L1Hs* open reading frame (p40 gene). We have done this for several reasons; (1) we have an anti-p40 antibody that is capable of detecting small quantities of the protein. Thus, we can easily assay cells for *L1Hs* expression. (2) Experience gained in this work will be very useful in placing the full-length *L1Hs* element into the same cells.

(a) Transfection of breast epithelial cells.

We have used three human, non-malignant breast epithelial lines in our work: HBL100, Hs587Bst, and MCF10A. Transfection protocols have utilized (a) calcium phosphate-DNA co-precipitation, (b) a DEAE-dextran-DNA mix, and (c) several liposome-DNA complexes (LipofectAMINE, LipofectACE, and Lipofectin, all sold by Gibco-BRL). Our initial experiments utilized a β-galactosidase-containing vector, since measurement of the enzyme activity in cell extracts is very sensitive. Using this vector, the HBL100 cells were completely refractory to transfection. In addition, colleagues have informed us that this cell line is refractory to transfection by electroporation. Similarly, we were unable to obtain transfected Hs587Bst cells. Control transfections utalizing COS cells were successful (COS is an SV40-transformed monkey kidney cell line that is easily and efficiently transfected). Thus, we have concentrated our efforts on the MCF10A cell line.

Transfection of MCF10A cells with the β-galactosidase-containing vector suggested that

transfection was possible, but the efficiency was low. Indeed, when we stained a population of transfected cells, it was apparent that less than 1% were actually taking-up DNA under our most optimal conditions. Nevertheless, this is certainly sufficient to give us stably transfected cells that express *L1Hs*, which is our goal. To obtain stably transfected cells we treat with a LipofectACE-DNA complex, then subject the cells, after an appropriate time interval, to a neomycin analog (G418) since the vector contains both the *L1Hs* p40 gene and a neomycin (neo) resistance gene. Neo resistant cells are cloned, grown in large numbers, and assayed for p40 expression.

(b) Expression of the *L1Hs* p40 gene in transfected cells.

We have placed the following plasmid-based constructs into the MCF10A cell line: RSV-p40, CMV-p40 and MMTV-p40. These vectors place the *L1Hs* p40 gene behind the Rous sarcoma virus promoter/enhancer, the cytomegalovirus promoter/enhancer and the mouse mammary tumor virus promoter/enhancer, respectively. All constructs proved to be capable of expressing the p40 protein, as shown by Western blotting extracts from transfected COS cells. In addition, we have placed the p40 protein into several retroviral vectors: LXSN, LNSX and Elneo [6,7]. The LXSN and LNSX vectors have two promoters, one for the neo gene and one for the p40 gene. The Elneo vector contains only a single promoter/enhancer. With this vector the neo and p40 genes can both be expressed due to mRNA splicing. Thus, complete transcripts express the p40 protein which is located at the 5' end of the mRNA, while spliced mRNAs express the neo gene product.

All of the tested constructs produced stable, drug resistant colonies after transfection into MCF10A cells. However, in no case was p40 detected. To date we have tested the following numbers of neo-resistant colonies: 6 Elneo, 20 LNSX, 7 RSV, 6 CMV and 8 MMTV. LXSN is still under development because of packaging problems.

We assume, but have not yet shown, that for vectors with two promoters the promoter and/or enhancer driving the expression of the p40 gene is inactivated, probably by methylation. The promoter/enhancer for the neo gene, which is under strong selection, is obviously still functional. Exactly why the Elneo construct produces no p40 is unknown. We have been informed by colleagues that in some cells virtually all mRNAs from this vector are spliced. Apparently MCF10A is such a line.

(c) The bicistronic expression vector pIRES1neo.

In October, 1996, Clonetech Laboratories, Inc. of Palo Alto, CA, introduced a new expression vector, pIRES1neo. This vector was constructed because many laboratories have experienced difficulties, similar to ours, expressing certain genes in some cell types. The vector has the following properties: transcription is driven by a CMV immediate-early promoter/enhancer. Following the CMV region is a multiple cloning site and then an internal ribosome entry site (IRES) derived from the encephalomyocarditis virus. Following the IRES region is a neo resistance gene. The transcript produced by the construct contains the gene of interest and the neo gene on one mRNA, and both can be translated due to separate ribosome binding sites. Thus, G418 resistant cells should, barring deletion, also express the gene of interest.

We have cloned the *L1Hs* p40 gene into this construct (plasmid pBC-1) and transfected it

into MCF10A cells using a new transfection protocol. This new transfection protocol uses a reagent, GF1, sold by Qiagen. GF1 is a polycation with colloidal properties and is claimed to be very efficient in transfecting a broad range of cell types. We used this reagent in the hopes of increasing the transfection efficiency of the MCF10A cell line, which, in our hands, transfects poorly with other reagents. We noticed no great improvement in transfection efficiency with the GF1 reagent *vis-a-vis* other reagents. However, Western blotting of proteins from G418 survivors following transfection with pBC-1 demonstrated that small amounts of p40 protein were present. In order to increase the amount of protein we took advantage of the fact that the CMV immediate-early promoter/enhancer responds to phorbol esters [8]. Treatment with TPA caused a massive increase in the amount of p40, as expected (Fig. 1, lanes 4 and 5; although not visible in this digitized figure, lanes 2 and 3 have a small amount of p40).

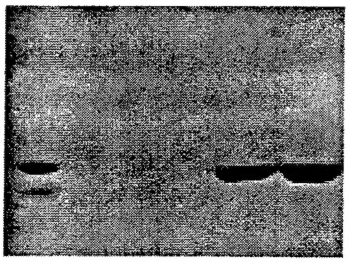

Fig. 1: Expression of p40 in MCF10A cells. The p40-containing vector, pBC-1, was introduced into cells and G418 resistant survivors were isolated and treated with various agents:

Lane 1: Control proteins from the T47D breast cancer cell line.

Lane 2: 200 nM 4α-phorbol-13,13-dideconoate (4αPD, a non-active phorbol ester analog) for 30 min., then growth for 24 hr.

Lane 3: no treatment.

Lane 4: 50 nM 12-O-tetradecanoyl-phorbol-13-acetate (TPA, an active phorbol ester) for 30 min., then growth for 8 hr.

Lane 5: 50 nM TPA, for 30 min., then growth for 24 hr.

We are currently cloning our "tagged" *L1Hs* element into this construct, which will then be transfected into MCF10A cells. Although some expression will occur from the uninduced CMV promoter, we feel that it may be of interest to modulate the amounts by inducing the promoter to varying extents. Since we do not wish to treat the cells with TPA, we are investigating other agents that might be more suitable than phorbol esters since there is a good deal of "cross talk" among protein kinase cascade pathways [9].

To use the pIRES1neo vector will require the removal of the *L1Hs* polyadenylation signal (this will be done with the *Acc*I restriction enzyme which has a site 50 bp from the end of the element). The fact that the mRNA now also contains the neo gene 3' of the *L1Hs* element will probably not interfere with the experiment since it has been shown that *L1Hs* elements with 3' extensions of up to 490 bp can still retrotranspose [10,11]. In fact, it is possible that we can use this extention to our advantage since we will now have the IRES plus neo gene sequences to use as a tag. Since retrotransposed *L1Hs* inserts are highly truncated [11], we can distinguish transfected and retrotransposed copies of the construct by size.

Conclusions

A successful execution of the goals of the grant require that a full-length, transpositionally competent *L1Hs* element be placed into a non-malignant breast epithelial cell. After a number of failures, we have found a vector that appears suitable for our purposes. This vector produces a bicistronic mRNA and allows the overexpression of cloned genes by transient activation with phorbol esters and possibly other agents. Thus, we now feel that Aim 1 of the grant (vector construction and expression in non-malignant breast cells) will be accomplished soon.

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